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BRIEF COMMUNICATION

Rapid extra-/intracellular biosynthesis of gold nanoparticles by the fungus *Penicillium* sp.

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Abstract In this work, the fungus *Penicillium* was used for rapid extra-/intracellular biosynthesis of gold nanoparticles. $AuCl_4^-$ ions reacted with the cell filtrate of *Penicillium* sp. resulting in extracellular biosynthesis of gold nanoparticles within 1 min. Intracellular biosynthesis of gold nanoparticles was obtained by incubating $AuCl_4^-$ solution with fungal biomass for 8 h. The gold nanoparticles were characterized by means of visual observation, UV–Vis absorption spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX). The extracellular nanoparticles exhibited maximum absorbance at 545 nm in UV–Vis spectroscopy. The XRD spectrum showed Bragg reflections corresponding to the gold nanocrystals. TEM exhibited the formed spherical gold nanoparticles in the size range from 30 to 50 nm with an average size of 45 nm. SEM and TEM revealed that the intracellular gold nanoparticles were well dispersed on the cell wall and within the cell, and they are mostly spherical in shape with an average diameter of 50 nm. The presence of gold was confirmed by EDX analysis.

Keywords *Penicillium* · Extra-/intracellular · Biosynthesis · Gold nanoparticles

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Introduction

Nanostructured materials have been attracting considerable attention because of their unique physical and chemical properties, which give them potential for important applications in many fields. The effective and reliable synthesis of nanomaterials is essential for their advancement. There is a growing tendency to develop green chemistry processes for the synthesis of nanomaterials. Researchers in nanosynthesis are increasingly turning to the natural systems of organisms for inspiration, which could provide exciting possibilities for the development of nanosynthesis (Mandal et al. 2006; Mohanpuria et al. 2008). Some well-known examples demonstrating that living organisms synthesize advanced nanomaterials include magnetotactic bacteria that synthesize magnetic nanocrystals (Dickson 1999) and nanobacteria that propagate calcifying nanoparticles (Kajander 2006). Using microorganisms for nanosynthesis is an emerging technique at the intersection of

nanotechnology and biotechnology. This area is of great appeal to academic and development researchers and also renews interest in nanosynthesis.

Among the microorganisms considered as potential eco-friendly nanofactories for the synthesis of metal nanoparticles, bacteria were studied early in the field and have since received the most attention (Joerger et al. 2001). To break this tradition, the National Chemical Laboratory (NCL) in Pune, India, embarked on a highly interdisciplinary program. They investigated whether eukaryotic organisms such as fungi may be used instead of bacteria to prepare nanoparticles. In a two-year effort, about 200 genera of fungi were investigated for this purpose. From this extensive screening study, two genera of fungi, *Verticillium* sp. (Mukherjee et al. 2001a, b) and *Fusarium oxysporum*, were found to produce nanoparticles. The latter displayed the ability to synthesize gold and silver nanoparticles extracellularly (Mukherjee et al. 2002; Ahmad et al. 2003a; Durán et al. 2005). Compared with bacteria, fungi are considered as a source for the higher productivity of nanoparticles with the advantage of secreting much larger amounts of proteins (Sastry et al. 2003).

The nanoparticles synthesized by microorganisms in previous reports are mainly intracellular, on the cell wall or within the cell such as cytoplasmic membrane, the periplasmic space, the cytoplasm, and the nucleus. However, for further analysis and some practical applications, additional steps may be required for isolating the pure nanoparticles from the biomass by ultrasonication (Kalimuthu et al. 2008, 2009; Ganesh and Gunasekaran 2009) or reaction with suitable detergents (Nangia et al. 2009) or lysis buffer (Samadi et al. 2009). This makes downstream processing difficult and time-consuming, negating the purpose of developing a simple and cheap method of nanosynthesis. Thus, it would be more practical if the nanoparticles are formed extracellularly, i.e., directly in the reaction solution. The extracellular synthesis of gold nanoparticles has been reported recently by the bacteria *Pseudomonas aeruginosa* (Husseiny et al. 2007) and *Rhodopseudomonas capsulata* (He et al. 2007, 2008), the actinomycete *Thermomonospora* sp. (Ahmad et al. 2003b), the fungi *Trichothecium* sp. (Ahmad et al. 2005), and *Colletotrichum* sp. (Shankar et al. 2003). Silver nanoparticles were also synthesized extracellularly by the bacteria *Enterobacter cloacae* (Shahverdi et al. 2007), *Escherichia coli* (Shahverdi et al. 2007; Gurunathan et al. 2009), *Bacillus licheniformis* (Kalishwaralal et al. 2008), *Klebsiella pneumonia* (Shahverdi et al. 2007; Mokhtari et al. 2009), silver-tolerant yeast strain MKY3 (Kowshik et al. 2003), the fungi *Aspergillus fumigatus* (Bhainsa and D'Souza 2006), *Penicillium* sp. (Sadowski et al. 2008), *Cladosporium cladosporioides* (Balaji et al. 2009), *Penicillium fellutanum* (Kathiresan et al. 2009), *Staphylococcus aureus* (Nanda and Saravanan 2009), *Alternaria alternate* (Gajbhiye et al. 2009), *Phoma glomerata* (Birla et al. 2009), *Trichoderma viride* (Fayaz et al. 2010), *Coriolus versicolor* (Sanghi and Verma 2009), and *Fusarium solani* (Ingle et al. 2009). In extracellular biosynthesis, nanoparticles are usually synthesized through the contact of precursor ions with the biomass (Mukherjee et al. 2002; Ahmad et al. 2003a, b, 2005; Durán et al. 2005; He et al. 2007; Shankar et al. 2003; Kowshik et al. 2003), the cell filtrates separated after incubation of the biomass in sterile water (Mukherjee et al. 2002; Ahmad et al. 2003a; Durán et al. 2005; He et al. 2008; Bhainsa and D'Souza 2006; Sadowski et al. 2008; Kathiresan et al. 2009; Gajbhiye et al. 2009; Birla et al. 2009; Fayaz et al. 2010; Ingle et al. 2009) or the culture supernatants obtained from the inoculated culture (Husseiny et al. 2007; Shahverdi et al. 2007; Gurunathan et al. 2009; Kalishwaralal et al. 2008; Mokhtari et al. 2009; Balaji et al. 2009; Nanda and Saravanan 2009; Sanghi and Verma 2009).

Compared with the conventional physical and chemical methods used for nanoparticle synthesis, the major drawback of biosynthesis using microorganisms may be the long reaction time which can be several days. If nanoparticles can be synthesized more rapidly, the biosynthetic procedures will have greater commercial viability. Extracellular synthesis of gold or silver nanoparticles by a biomass is a relatively slow process with contact times ranging from 24 (Kowshik et al. 2003) to 120 h (Ahmad et al. 2003b). However, Bhainsa and D'Souza (2006) reported the synthesis of silver nanoparticles just 10 min after silver ions came into contact with the cell filtrate of *Aspergillus fumigatus*. Shahverdi et al. (2007) presented the rapid synthesis of silver nanoparticles within 5 min using culture supernatant of *Enterobacteria*. Nevertheless, there are relatively complicated components in culture supernatants, including considerable amounts of residual culture medium and its metabolites. Cell filtrates have a large

quantity of extracellular secretions in a relatively pure state and are free from other cellular proteins associated with the biomass (Sastry et al. 2003). The enzymes or agents responsible for the synthesis of nanoparticles can easily be isolated from the cell filtrate. It is important to ascertain the mechanism of nanosynthesis by microorganisms (Durán et al. 2005; Kumar et al. 2007) so that better control over the size, shape, or polydispersity of the nanoparticles can be achieved (He et al. 2008; Fayaz et al. 2010).

In this article, the fungus *Penicillium* sp. strain 1-208 was used to synthesize gold nanoparticles. The cell filtrate of this fungus was used to develop an extracellular process for the synthesis of gold nanoparticles. Gold nanoparticles were observed within 1 min after $AuCl_4^-$ solution was added to the cell filtrate. To our knowledge, this is the first report showing such rapid synthesis of gold nanoparticles by microorganism. In addition, intracellular biosynthesis of gold nanoparticles was achieved by incubating $AuCl_4^-$ solution with the fungal biomass for 8 h. Extra-/intracellular synthesis of gold nanoparticles by the same fungus is other highlight of this work.

Experimental details

Reagents

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) from Sinopharm Chemical Reagent Co., Ltd. was used as received. All other reagents were of analytical grade. Unless otherwise stated, deionized water was used in all of the experiments.

Fungal strain and growth conditions

The fungal strain *Penicillium* sp. 1-208 was used in this study. To prepare the cell filtrate and biomass for biosynthesis experiments, the fungus was grown aerobically in 250 mL of a liquid medium containing 1% starch, 0.25% $(NH_4)_2SO_4$, 0.3% K_2HPO_4, 0.5% tryptone, 0.02% $MgSO_4 \cdot 7H_2O$, 0.00255% $FeSO_4 \cdot 7H_2O$, and 0.013% $CaCl_2$ with pH of 5.5. The flasks were inoculated, incubated at 28 °C, and agitated at 180 rpm.

Intracellular biosynthesis of gold nanoparticles

The biomass was harvested after growth for 3 days in culture medium by filtration through a 4-layer sterilized carbasus. The biomass was then thoroughly rinsed with sterile deionized water to remove any medium components. The harvested biomass (0.5 g of wet mycelia) was resuspended in 10 mL of 1 mM $HAuCl_4$ solution and then placed in a shaker for intracellular biosynthesis.

Extracellular biosynthesis of gold nanoparticles

2 g of fresh biomass was added to 20 mL of sterile deionized water and incubated at 28 °C with agitation at 180 rpm for 3 days. After incubation, the cell filtrate was obtained by centrifugation at 6,000 rpm for 5 min. To synthesize gold nanoparticles, $HAuCl_4$ solution was added dropwise to 10 mL of the cell filtrate with a final concentration of 1 mM under stirring.

Apparatus and measurements

A UV–Vis spectrophotometer (Unico UV-2102 PCS, China) was employed for absorption spectra measurements of the extracellularly synthesized nanoparticles. In the kinetic study carried out on Biomate 3 spectrophotomer (Thermo Scientific, USA), 545 nm was selected as the test wavelength with the interval time of 5 s and the total run time of 5 min. The gold nanoparticles were also investigated using transmission electron microscopy (Hitachi H-500, Japan). Samples for TEM characterization were prepared by placing a drop of the solution onto a carbon-coated copper grid which was then dried at room temperature. XRD analysis of the gold nanoparticles, which were prepared by casting the solution on a glass substrate, was carried out on an X-ray diffractometer (Rigaku D/MAX 2500 V, Japan).

SEM and EDX measurements of the fungal cells after the formation of the gold nanoparticles were carried out on a scanning electron microscopy (Hitachi S-3400 N, Japan). The samples were prepared by casting the nanoparticle-decorated fungal cell solution on a glass substrate. Since the images obtained for the as-prepared samples were of good quality, sputtering of gold or carbon film on the samples was not required. The fungal cells without

interacting with $HAuCl_4$ were measured as blank control, in which case the carbon film was sputtered. TEM was also applied to analyze the intracelluar gold nanoparticles. The ultrathin sections of the fungal cells interacting with $HAuCl_4$ were prepared according to the procedure adapted from the previous reports (Mukherjee et al. 2001a; Anshup et al. 2005). In brief, the gold nanoparticle–fungus composites were fixed in 3% gluteraldehyde. After postfixation in 1% osmium tetroxide, the composites were dehydrated in graded series of ethanol and infiltrated and embedded in Epon 812 and polymerized at 60 °C for 3 days. Ultrathin sections were cut in an ultramicrotome (Leica Ultracut UCT, Germany). Finally, the sections were stained with uranyl acetate for the TEM analysis.

Results and discussion

Inset a of Fig. 1 shows the pale yellow cell filtrate from *Penicillium* sp. The solution turns purple (Inset b of Fig. 1) after addition of $HAuCl_4$ solution to the stirred cell filtrate. Considering that the $HAuCl_4$ solution and the cell filtrate are both light in color, the change in color to the characteristic purple of gold nanoparticles clearly indicates their formation in the reaction solution (Link and El-Sayed 1999; Mukherjee et al. 2002; Kalimuthu et al. 2009). Visual observation of the biosynthesis process shows that it occurs rapidly. The color of the reaction solution changes to purple without further increase in intensity within 1 min. Compared with the previously reported times from several hours to several days for the extracellular biosynthesis of gold nanoparticles (Husseiny et al. 2007; Ahmad et al. 2003b), the time required in this work is significantly shorter.

UV–Vis absorption spectroscopy was also used to measure the biosynthesis of gold nanoparticles. The UV–Vis spectrum illustrated in Fig. 1c shows no evidence of an absorption peak in the region of 300–800 nm for the cell filtrate from *Penicillium* sp. 1-208. After the $HAuCl_4$ solution has reacted with the cell filtrate, a well-defined absorption peak at ca. 545 nm appears in Fig. 1d that corresponds to the wavelength of the surface plasmon resonance of gold nanoparticles (Mukherjee et al. 2002; Mulvaney 1996). Various reports have established that the resonance peak of gold nanoparticles appears around this region, but the exact position depends on a number of factors (Link and El-Sayed 2000; Nair and Pradeep 2002). The absorption peak is sharp, which indicates little aggregation of the nanoparticles in solution.

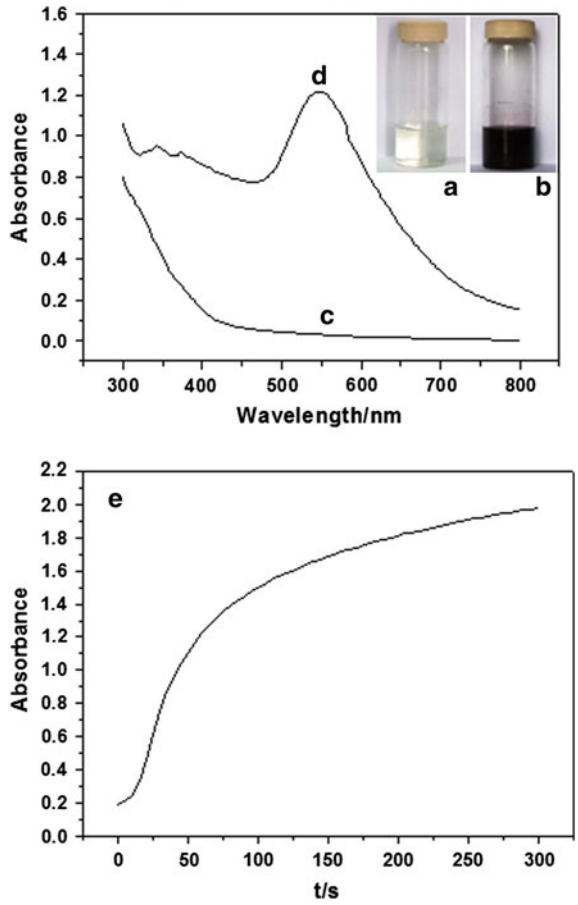

Fig. 1 The *inset* shows the color of the cell filtrate from *Penicillium* sp. 1-208 before (**a**) and after (**b**) reaction with 1 mM $AuCl_4^-$ solution. The corresponding UV–Vis spectra are shown in **c** and **d**, respectively. **e** The absorbance change against reaction time during the cell filtrate interacting with 1 mM $AuCl_4^-$ solution

The kinetic response of nanoparticles synthesis was tested by measuring the time evolution of the absorbance at the fixed wavelength (Lee et al. 2009). According to the result in Fig. 1d, 545 nm was selected for the study. The reaction solution was mixed quickly in the spectrophotometer cell before the test, and then was tested under static condition. The analysis result is illustrated in Fig. 1e, in which the measuring absorbance corresponds to the gold nanoparticles produced in the reaction process.

Observed from the result, the absorbance increases very rapidly in the initial stage to around 1 min, which indicates the rapid synthesis of gold nanoparticles, then a slow increase is followed. This test and the visual observation indicate that the formation of gold nanoparticles can occur in a short time of 1 min after the $HAuCl_4$ solution came into contact with the cell filtrate. However, the reaction would be stirred to occur in a dispersed system for a technical synthesis.

X-ray diffraction measurement over the whole spectrum of 2θ values from 35 to 80° can provide information about the crystalline nature of the gold nanoparticles (Fig. 2). Four prominent Bragg reflections corresponding to the (111), (200), (220), and (311) orientations are observed in the diffraction pattern, which are consistent with the features of bulk gold with a face-centered cubic (fcc) structure and also agree with those reported for gold nanocrystals (Ahmad et al. 2005; Leff et al. 1996; Deplanche and Macaskie 2008; Du et al. 2007). The results confirm the presence of gold nanocrystals formed by the extracellular filtrate from *Penicillium* sp. and show that they are of preferential (111) orientation.

Information about the size distribution and morphology of gold nanoparticles synthesized by the cell filtrate may be obtained from TEM measurements. Two TEM images are displayed in Fig. 3, which are recorded from different regions of the gold nanoparticles deposited on a carbon-coated copper grid. Observed from Fig. 3a, the gold nanoparticles are scattered and most of them present in spherical. An enlarged micrograph is shown in Fig. 3b, in which discrete gold nanoparticles can clearly be discerned. The gold nanoparticles must be stabilized by some agents in the extracellular secretions from the fungus which prevent the aggregation of gold nanoparticles. Statistical analysis result of the size distribution from the TEM images is shown in Fig. 3c, which shows that most of the particles are in a size range from 30 to 50 nm. The average diameter is estimated to be 45 nm.

It is clear that the extracellular filtrate prepared from *Penicillium* sp. 1-208 can be used for the extracellular biosynthesis of gold nanoparticles. Specific agents in this filtrate play roles in reducing the $AuCl_4^-$ ions, and the subsequent formation and stabilization of the gold nanoparticles. Research to further understanding this intricate biosynthesis process and the responsible agents will be undertaken in the future. In this study, the approximate mole concentration of the synthesized nanoparticles can

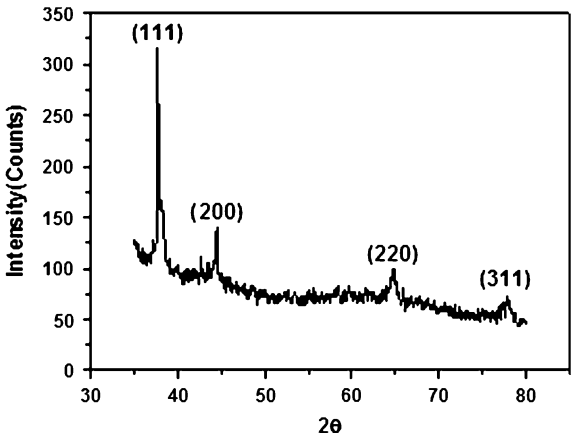

Fig. 2 XRD pattern of gold nanoparticles deposited on a glass slide. *Labeled peaks* correspond to the characteristic diffraction peaks of elemental Au

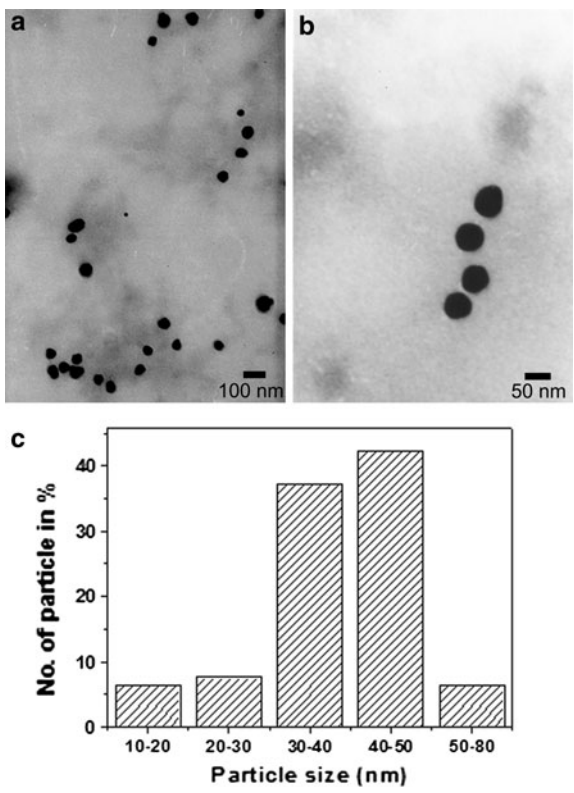

Fig. 3 TEM micrographs of the extracellular gold nanoparticles at magnifications of **a** 45,000× and **b** 110,000×. **c** Particle size distribution histogram determined from TEM images

be given on the assumption that all the golds in solution are reduced to form nanoparticles (Link and El-Sayed 1999). Acoording to the size of nanoparticles from TEM analysis and the density of bulk fcc gold, the concentration of the synthesized gold nanoparticles is estimated to be in the range of 10^{-9}–10^{-10} M for 45 nm particles. In the previous report (Mukherjee et al. 2002), the cell filtrate from 10 g of biomass immersed in 100 mL of water interacted with 100 mL of 2 mM $HAuCl_4$ solution, which synthesized spherical and triangular gold particles in the size range of 20–40 nm. In another report (He et al. 2008), the cell filtrate obtained from 1 g of biomass incubated in 10 mL of water interacting with 0.25 mM $HAuCl_4$ formed exclusive spherical gold nanoparticles with size ranging of 10–20 nm. When the concentration of $HAuCl_4$ was above 0.4 mM, the cell filtrate was insufficient relative to gold ions for the synthesis of nanoparticles, but formed gold nanowires with a network structure. Compared with these reports, the gold nanoparticles synthesized in this study have larger size range and average diameter. The cell filtrate shows a good activity for the synthesis of gold nanoparticles.

The fungus was incubated in culture for 3 days, then collected and rinsed with sterile-deionized water. The clean biomass was then exposed to a 1 mM solution of $HAuCl_4$ for 8 h. The formation of gold nanoparticles within the fungal cells changes the color of the fungus from milk white (Fig. 4a) to purple (Fig. 4b), the characteristic color of gold nanoparticles.

The presence of intracellular gold nanoparticles can be confirmed by SEM measurements. Figure 4c shows a number of filaments of fungal mycelia decorated with white spots after reaction with $AuCl_4^-$ solution. Figure 4d shows the image more clearly at higher magnification. Compared with Fig. 4e, which shows several blank filaments without decoration, we consider that the white spots are well-dispersed gold nanoparticles, which is consistent with the color observation in Fig 4a and b. There still are some white spots dispersed on the substrate. This illustrates that some of the gold nanoparticles are synthesized extracellularly by the small amount of extracellular secretions released into the solution from the fungus during the intracellular biosynthesis of gold nanoparticles.

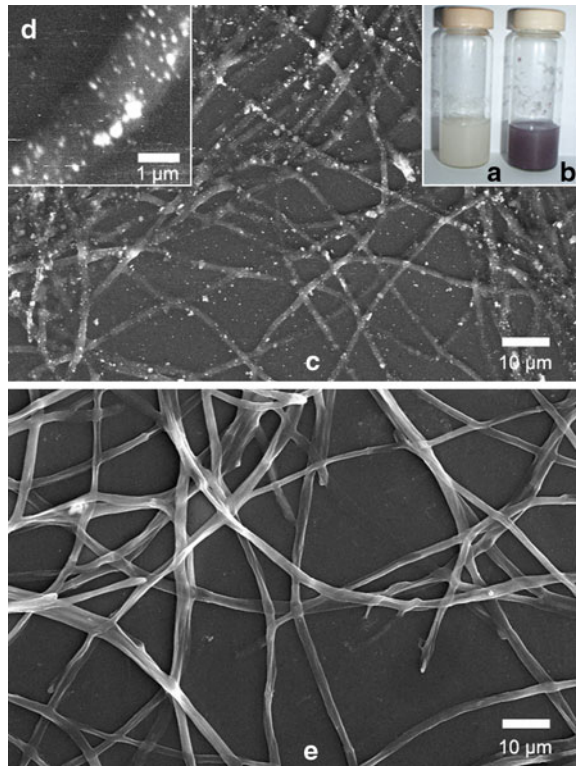

Fig. 4 The *inset* in the top right corner shows the pictures of *Penicillium* sp. biomass (**a**) in sterile water and (**b**) after reaction with 1 mM $AuCl_4^-$ solution. **c** SEM micrograph from *Penicillium* sp. 1-208 after reaction with 1 mM $AuCl_4^-$ solution. The *inset* in the top left corner (**d**) shows an SEM image at higher magnification. **e** SEM micrograph from *Penicillium* sp. 1-208 without reacting with $AuCl_4^-$ solution

The presence of strong signals consistent with elemental gold together with weak signals from C, N, O, Na, Si, Cl, and Ca atoms are observed in the EDX analysis of the synthesized nanoparticles (Fig. 5). Two peaks at about 2.15 and 9.70 keV show the presence of gold (Losic et al. 2005; Tseng et al. 2009). The C, N, and O signals may arise from proteins, enzymes, or the fungal biomass which are bound or near to the gold nanoparticles (Mukherjee et al. 2001a; He et al. 2008). The Cl signal indicates the presence of a small amount of $AuCl_4^-$ ions in the investigated region. The Na, Si, and Ca signals are likely caused by X-ray emission from the glass substrate used in the EDX analysis (Nangia et al. 2009).

Representative TEM images in Fig. 6 show the well-dispersed gold nanoparticles distributed within the mycelia of the *Penicillium* fungus. At low

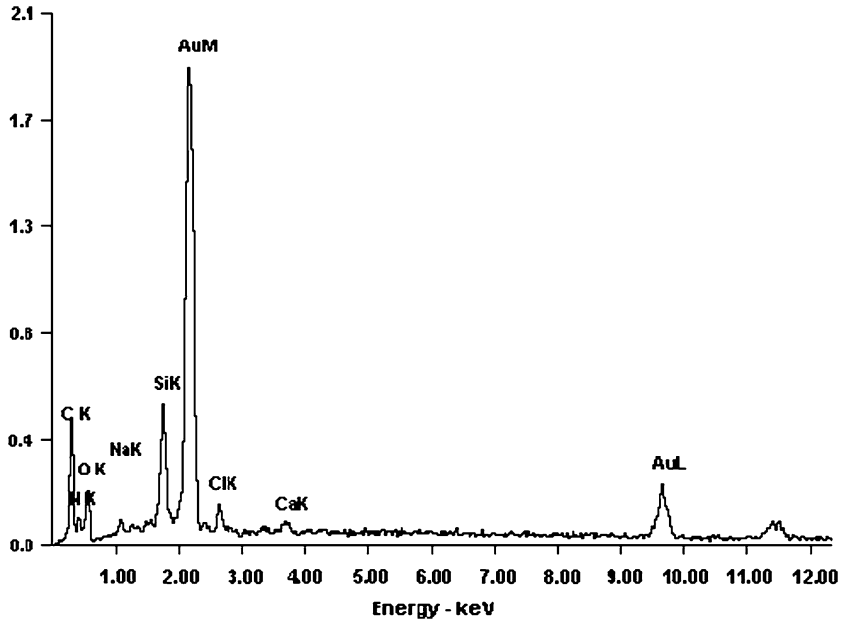

Fig. 5 EDX spectrum recorded from a film of the gold nanoparticle-fungus composite. Units on the Y-axis are in kilo counts

magnification (Fig. 6a), a black section in the middle of the image corresponds to the septum connecting two mycelia. The morphology and size of the gold nanoparticles are displayed more clearly in Fig. 6b at slightly higher magnification. Figure 6c shows the particle size distribution histogram determined from the TEM results. Observed from the figure, the as-prepared gold nanoparticles are mostly spherical in shape, with an average diameter of 50 nm.

To investigate the location of the gold nanoparticles in relation to the mycelia, TEM studies were carried out to analyze the ultrathin sections from fungal cells decorated with gold nanoparticles. The TEM results from two different regions are illustrated in Fig. 7 at the same magnification. The nanoparticles were found to be formed both on the cell wall as well as within the cell observed from Fig. 7a. In Fig. 7b, all gold nanoparticles were found to be within the cells. The result was consistent with the previous reports (Mukherjee et al. 2001a; Anshup et al. 2005).

The rapid extra-/intracellular biosynthesis of gold nanoparticles is realized by fungus *Penicillium* sp. 1-208. The difference between the two kinds of synthesis is the components which are responsible for the synthesis of the gold nanoparticles. Although the exact mechanism of biosynthesis has not been elucidated, we speculate that specific enzymes or

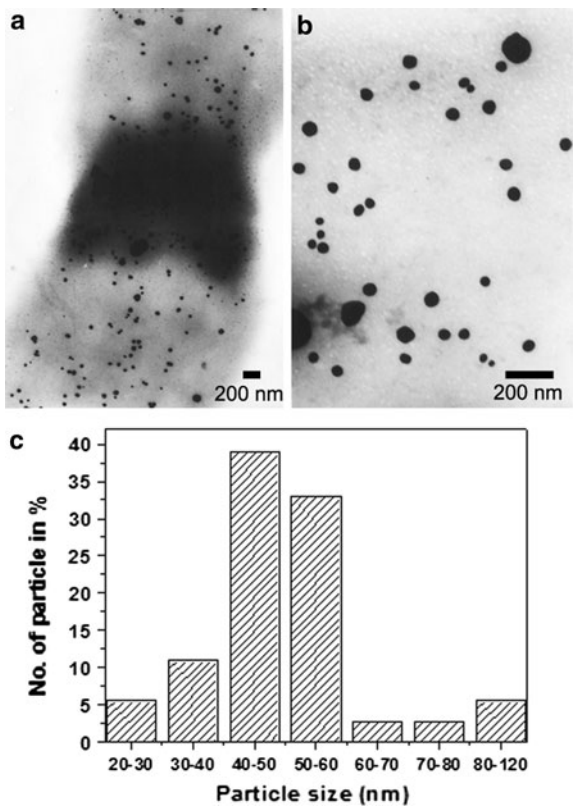

Fig. 6 TEM micrographs of the intracellular gold nanoparticles at magnifications of **a** 15,000× and **b** 42,000×. **c** Particle size distribution histogram of gold nanoparticles determined from TEM images

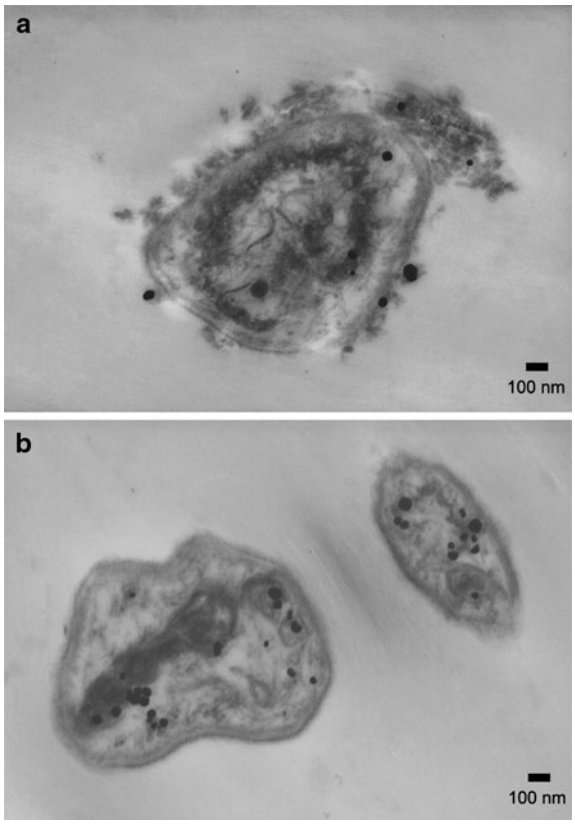

Fig. 7 TEM micrographs from ultrathin sections of the fungal cells decorated with gold nanoparticles, in which **a** and **b** are from two different regions at the same magnification of 25,000×

agents released by the fungus into the solution are responsible for the extracellular synthesis. Intracellular growth of gold nanoparticles may be caused by enzymes bound to the cell wall of the fungus or within the fungal cells. Thus, extracellular synthesis occurs in the cell filtrate from the fungus and intracellular synthesis occurs in the fungal biomass. The extra-/intracellular formation of gold nanoparticles by the fungus *Penicillium* sp. used in this study is a prominent ability among the many organisms studied for the synthesis of gold nanoparticles.

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